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XPS Analysis and Antibacterial Assay of Novobiocin Coating

Hardi Ys[a]* , Susan Semple[b] , Hans J. Griesser[c]

[a]*University of Tadulako, Palu, Indonesia*
[b]*School of Pharmacy and Medical Sciences-University of South Australia, Adelaide, Australia*
[c]*Mawson Institute- University of South Australia, Adelaide, Australia*

Abstract

Infection disease after biomedical implant surgery is often caused by unintentional contamination of micro-organism. Novobiocin is one of the most attractive antibiotic due to its high activity against gram positive bacteria and its relatively compatible to fibroblast cell line. Based on the molecular structure of novobiocin, it may be conjugated or coated onto material surfaces. The coating steps were plasma polymerisation, polymer grafting, and novobiocin conjugation. XPS analysis showed that the elements and high resolution of C 1s confirmed that the surface chemical had changed. Novobiocin, after conjugation, became more dominant in covering the surface. Antibacterial Assay to *Staphylococcus epidermidis* by using dead and live kit indicated the reduction of bacteria reached up to 97.27% and only 0.07% of live bacteria left. This result led to a conclusion that immobilised novobiocin may be considered as novel antibacterial coating that can prevent nosocomial infection.

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Keywords: novobiocin; plasma-polymerisation; antibacterial coatings; XPS analysis

1. Introduction

Infections following implant surgery or medically termed as nosocomial infection, is often caused by unintentional contamination of micro-organism during implanting device process. The micro-organism might be from air borne, medical device, or mistaken treatment circumstances. Once the bacteria contaminated the medical implant, it tries to do initial adhesion onto device surface via specific interaction between outside cells of microorganism and certain functional or molecular group on the substratum surface [1]. Post initial adhesion, bacteria tries to settle and protect itself by producing a matrix exopolimer known as polysaccharide intercellular adhesin

* Hardi Ys. Tel.: +62-812-82052595; fax: +62-451-422355.
E-mail address: hardiys@live.com

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(PIA), so called biofilm [2] which is not easy to detect in the routine diagnostics and has resistance to host defences and antibiotic therapy [3].

Protected by biofilm, bacteria are hidden and become undetectable by immune-system because the exopolymer matrix has the ability to interfere with the performance of macrophage phagocytic activity [4,5]. Antibiotics and disinfectants therapy are incapable of eradicating biofilms since one of the biofilm properties is its resistance to in-diffusion of such compounds. Once the medical implant is infected in the human body and biofilm formed, the consequences might be re-operation, osteomyelitis, amputation, or death [6].

Most nosocomial infections are caused by gram-positive bacteria that act to form biofilm [7]. These staphylococci bacteria are *S. aureus* and *S. epidermidis*. *S aureus* commonly attach to metal-biomaterial, bone-joint, and soft-tissue infection [8]. While *S. epidermidis* prefer to settle on polymer-medical device [9]. An actual example is central venous catheters (CVCs) that penetrate into the human body. This device will contact directly with the bloodstream, the surface is touched and coated by host proteins, such as albumin, fibrinogen, fibronectin, vitronectin and laminin. *S. aureus* and *S. epidermis* are able to attach onto protein layers of fibronectin, fibrinogen, vitronectin, and laminin. The interaction and attachment of staphylococci to protein layer are specific and multifactor [4,10].

Current strategies for avoiding biofilm formation are: making sure that the device must be sterile; decreasing microbial attachment; killing the biofilm formation with antibacterial agents; and removal of the device [4]. Furthermore, hindering the initial adhesion of biofilm formation by covering the material surface with antibacterial agents is a favoured strategy [6].

Antibacterial coating research applied in many ways, such as impregnation-prophylactic, impregnation, and coupling double bond of antibacterial. Impregnation-prophylactic investigation uses antibiotics such as gentamicin [11–13], ciprofloxacin [14], and vancomycin [15]. Meanwhile, impregnation antibacterial investigation uses nano silver (particle size 5-50nm) with certain concentrations loaded in to plain material is effective and nontoxic [16]. However, it would be great in preventing the silver detachment from implant to avoid the silver from spreading over around the tissue [17].

Coupling covalent bonding between functional active groups of antibacterial and material were applied such as utilising quaternary ammonium compounds (QACs) and furanone compounds [18–22]. The covalent bonding method in biomaterial research, presumably, will keep the consistency of immobilised antibacterial, prevent the antibacterial come off from surface, and keep away from interfering with another host cell function.

Novobiocin showed that it had antibacterial activity when it was coupling bonding covalent onto material activated surface [22]. It also has good compatibility with fibroblast cell that has key-role in stimulating soft tissue generation [23]. This paper reports the surface analysis by using XPS and in depth antibacterial activity of *S. epidermidis*.

2. Materials and methods

2.1. Coating preparation

The Coating preparation steps, as shown at Fig.1, described in this sub-section. Plasma polymerisation was performed in a custom-build reactor. This technology was first published in 1989 and it shows that it is a radio frequency glow discharge (rfgd) based technology [24]. For the plasma deposition, in brief, the samples were placed on the base electrode, and then the chamber was outgassed to approximately 0.02 torrs. The fresh batch of monomer liquid connected to the plasma chamber. This liquid transforms to vapour stream by passing chamber connector into the chamber at specific pressure of 0.2-0.25 torrs. After stabilizing the pressure at the desired level, the glow expulsion was produced by giving a radio frequency electric field at 14 up to 16 W for 4-5 minutes. The monomer was left to flow into the chamber for another 10 minutes to assess in quenching free radicals in order to reduce the reaction of active free radicals left over after plasma treatment with atmospheric oxygen. The pressure in the reactor chamber was brought back to atmospheric pressure by bringing in air through another inlet valve. Plasma polymerisation of the monomer *n*-propionaldehyde was used for depositing the first layer onto Perfluorinated poly(ethylene-co-propylene) polymer (Teflon® FEP 100 Type A: 12.7mm wide, 25μm thick, Dupont, USA); a thin film of plasma polymer enriched with carbonyl groups deposited on the FEP, so called FEP-Ald slide.

Polyallylamine (PAA; MW 1500, Aldrich) grafted to FEP-Ald slide used *schiff bases reaction*, the slide was introduced to PAA (12 mg/ml in aqueous) 1 ml and sodium cyanoborohydride ($NaCNBH_3$; ≥95%, Fluka) (12 mg/ml in aqueous) 1ml. The reaction was kept in room temperature for overnight. Next, the slides were rinsed three times with the MilliQ water.

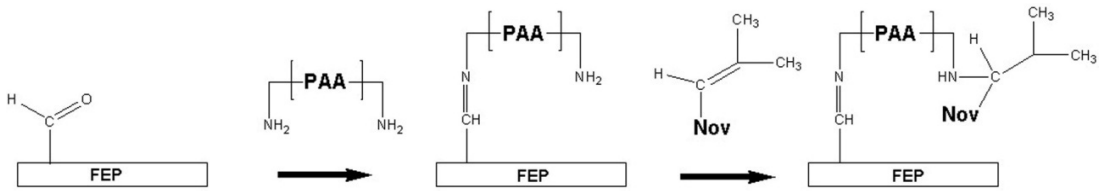

Fig. 1. Preparation steps of coating antibacterial on the surface [22]

The slide, after PAA being grafted, was put in round bottom flask (10 ml), and then, 3 ml of tetrahydrofuran (THF; Chem supply, SA) which contains 57 mg of Novobiocin (Sigma) and 27 mg of Mercury (II) Nitrate (Merck) were introduced into the flask. The flask's circumstance kept in Nitrogen gas condition. The flask cap was closed tightly, then, the flask was shaken in enclosed temperature-controlled shaker unit (4000 series, Bioline®, Adelab Scientific) for 24 hours with condition 120 rpm and 60^{o}C. Afterwards, the solution inside the flask was removed and replaced with 3 ml of $NaBH_4$ (3mg/ml in 10% NaOH). Next, the flask was put in the incubator for 18 hours at 120 rpm and 25^{0}C. The latter reaction need to be washed with milliQ water and shaken vigorously three times using vortex.

2.2. Physical analysis

XPS Analysis; AXIS Ultra DLD spectrometer (Kratos Analytical) was used for this XPS analyses and with the help of a monochromatic Al Kα source at a power of 130 W (13 kV x 10 mA). Energy pass for both survey spectra and spectra from individual element were 160 eV and 20 eV, while during analyses, the total pressure in the main chamber was $<10^{-8}$ mbar. The identification of elements present on the surface was from survey spectra. The identification was done after each modification stage in the immobilisation sequence. Finding out the relevant integral peak intensities used a non-linear Shirley-type background and applied the sensitivity factors which were supplied by the instrument manufacturer. This instrument also facilitated for calculating the atomic concentrations of all elements. Throughout the analyses process, charging effects of the samples were corrected using a value of 285.0 eV for the binding energy of the main C 1s component coming up from neutral hydrocarbon (CH_x) [25]. The XPS results of the coating multilayer structures are presented as atomic concentration in (%). Per surface modification, no less than three different sample locations were analysed and the average was considered.

XPS Spectra Analysis; Component spectra from different chemical species (survey method of XPS analyses) and bonding pattern (high resolution) of C 1s were measured using CasaXPS software. The curve's line shape was considered to be Gaussian-Lorentzian with a 30% Lorentzian component.

2.3. Microbial analysis

Microorganism: The microorganism used was *Staphylococcus epidermidis* strain ATTCC 35984 which was stored as stock culture at -80^{0}C. To keep the culture fresh for daily usage, the stock culture was rejuvenated by plating on blood agar and kept at 4^{o}C, and replating was done every one to two weeks.

Bacterial assay, staining, and analysis: The immobilised antibacterial slide was put in 12-wells plate (disposable cell culture, Nunclon ™ Surface, Denmark), then, the broth culture (2 ml) was introduced into each well. Broth culture evolved from *Staphylococcus epidermidis* ATTCC 35984 10^{-7} CFU/ml in Trypton Soy Broth (TSB) + 0.25 glucose. Then, the well plates containing culture were divided in two groups and were incubated at 37^{0}C for one hour and four hours. Then, the further preparations were: *Washing and Fixing*, broth culture in 12-wells plate was removed and washed with 2 ml PBS twice. Thus, the slide in the wells was immersed in 2 ml of 10% Formaldehyde saline for 10 minutes to fix the bacterial attachment on the slide surface; *Staining and Transferring,* after the fixing

reagent was disposed, 2 ml of the staining reagent (Bact Light LIVE/DEAD) was introduced into the wells and was shaken gently with approximately 10 stokes/ minute for 15 minutes, then using forceps, the stained slide was immersed into saline liquid and put onto the glass slide surface; *Covering and Observasion*, the top of slide's surface was wetted by mounted oil and covered with cover glass. Later, the slide was put underneath Fluorescence microscope (Olympus BX 40); *Analysis,* analysis was using AnalySis Five Olympus Imaging Software.

3. Results

3.1. Coating and surface analysis

The component spectra of immobilisation steps were analysed by XPS. Each steps of immobilisation was expected to have changes in surface character. The first layer (Ald), in which FEP as the base material, was produced by propionaldehyde vapour via plasma polymerisation. The second layer (Ald-PAA) was by PAA grafted and the third layer (Ald-PAA-Nov) was by aminomercuration-demercuration method.

XPS spectra analysis was using survey and high resolution of C 1s method. On the component spectra as the survey method, the surface changing from first layer to second layer can be detected by the existence of N 1s spectra (BE 397.5 eV). The Na 1s (BE 1068 eV) was detected on the third layer while it has none on the first and second layers.

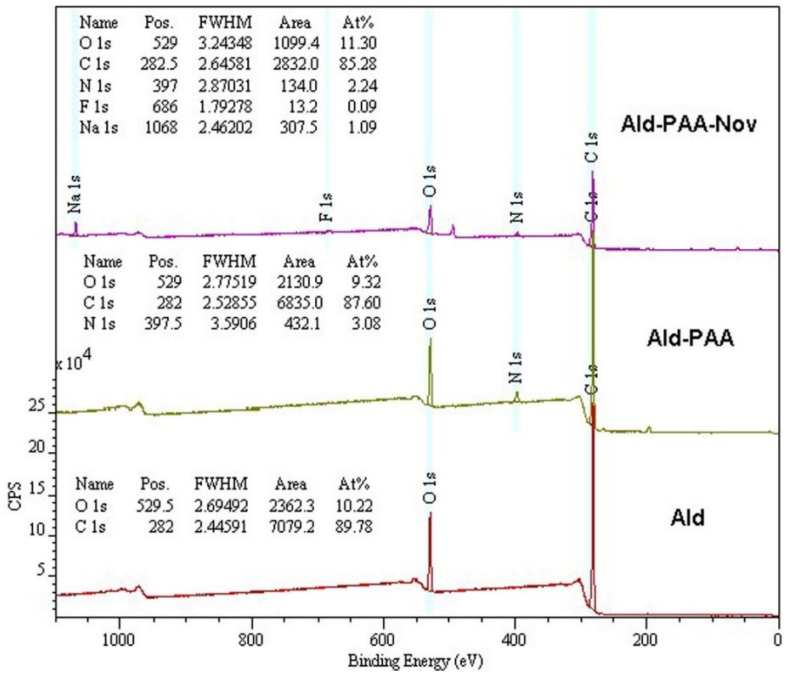

Fig. 2. Comparison layer by layer of component spectra from each steps immobilisation

High resolution C 1s identified the atomic bond of the surface. This method supports and confirms the survey of component spectra. Comparison layer by layer showed that the percentage area of C-N and C-O (BE 286.4 eV) of the second layer (Ald-PAA, 15.55%) is bigger than the first layer (Ald, 11.21%). It means that C-N composition increased, while, on the first layer the C-N bond did not exist because propional dehyde plasma polymerisation did not use the compound that contained C-N bond. The third layer (Ald-PAA-Nov), the percentage area of C-N and C-O decreased to become 10.90% (fig.3). Presumably, the amine functionalised group reacted by bonding covalently with Novobiocin compound, and also, novobiocin became more dominant in covering the surface. In addition, the existence of aromatic broad confirmed that novobiocin was well attached on the surface.

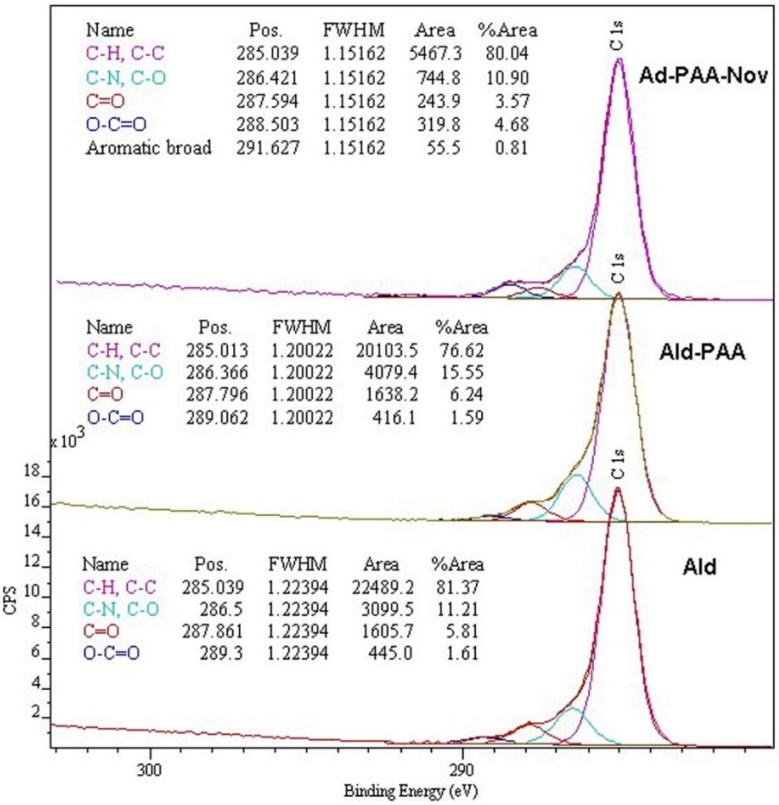

Fig. 3. Comparison layer by layer of high resolution C 1s from each steps immobilisation

3.2. Antibacterial and initial adhesion

Antibacterial activity of the immobilised novobiocin was tested using a bacterium that caused biofilm, *Staphylococcus epidermidis* ATTCC 35984. The first hour after incubation, many spots were detected on both samples, visually, spots of Ald-PAA were much more than the spots of Ald-PAA-Nov (fig. 4A and B). However, four hours after the incubation, significant change becomes obvious. Statistically, the reduction percentage before and after novobiocin immobilisation at the forth hour reached 97.21 % of total bacteria (fig. 4C and D).

4. Discussion

Physical analysis by XPS uses Al monochromatic X-ray source to produce photon. Modern XPS device mostly uses this source rather than achromatic sources such as Mg and Al. Monochromatic source produces a X-ray line many folds narrower than achromatic source. The achromatic Al K alpha $_{1,2}$ line produces a line width of <0.9 eV, whereas monochromated line is < 0.26 eV. In addition, the potential bombardment of filament is close to earth potential [26].

The analysis result of layer by layer toward the surface immobilised indicated that the spectra patterns were changing by each step of immobilisation. The pattern component spectra and high resolution C 1s curve's changes correlated with each step treatment (Fig. 2 and 3). The difference of typical layer by layer implied that the treatment works well. Unfortunately, the F 1s signal emerged on the Ald-PAA-Nov layer. This signal came from the FEP as the base material (data not shown). The appearance of spectra a signal from F 1s gave negative effect to surface material immobilised performance, presumably, this spectrum was detected due to some delaminating during immobilisation.

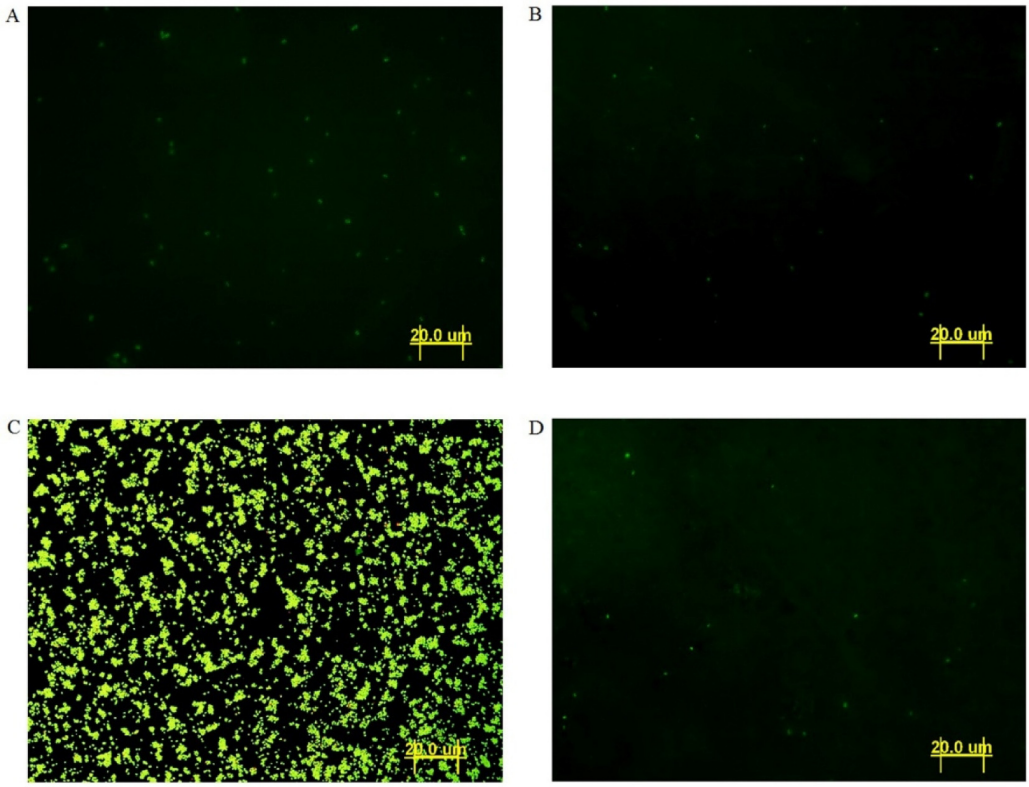

Fig. 4. Fluoresence imaging of S. epidermidis ATTCC 35984 attached on the surface; A and B are the first hour incubation, C and D are forth hour incubation. A and C are Ald-PAA layer whereas B and D are Ald-PAA-Nov layer

The relationship of physical analysis with the microbiological assay is that the existence of the elements or functional groups of novobiocin were detected in the XPS spectra after immobilisation, and then presumably, this existence correlated positively toward assay result in inhibiting bacteria activity.

Novobiocin as a promising anti-staphilococcal agent was discovered in 1955, produced by *Streptomyces spheroides* and *Streptomyces niveus* and investigated intensively at the time when penicillin resistant widespread [27]. Novobiocin is an aminocoumarin antibiotic which has ability to inhibit competitively DNA gyrase (also known as bacterial topoisomerase II) through blocking the ATP binding. It will then hinder DNA replication and thus bacteria reproduction. In addition, it is much more effective in inhibiting prokaryote than eukaryote topoisomerase II [28].

Furthermore, novobiocin has an impressive MIC; with MICs $\leq$ 1 ug/ml can kill 86.7% of sixty clinical isolates of oxacillin-resistant *Staphilococcus aureus*, while 100% of the bacteria can be killed with MICs $\leq$ 8 ug/ml. In addition, combining novobiocin into rifampin prevents the resistant emergence, since resistant increases common pathogen [29]. In its chemical structure, novobiocin has three rings: A is a prenylated 4-hydroxybenzoic acid; B is a substituted coumarin; and C is a noviose sugar (Fig. 6). The aminocoumarin on the ring B is supposed to be the active side; therefore, the asymmetrical double-bond on the ring A is assumed to be the inactive side of chemical structure of novobiocin [30–32].

Aminomercuration-demercuration method was used to immobilize double bond of novobiocin onto material surface. This method used mercury ion as the catalyst [33]. Addition of functional amine from surface material to assymetrical double bond of novobiocin, so called, immobilisation novobiocin on the surface shown that the mercury ion spectra was not detected by XPS. Mercury spectra is usually found at around BE 800 (Hg 4s), 677 (Hg 4p1/2), 571 (Hg 4p and Hg 4p3/2), 379 (Hg 4d3/2), 360 (Hg 4d and 4d5/2), 120 (Hg 5s), 103 (Hg 4f5/2), 99 (Hg 4f and 4f7/2), 81 (Hg 5p1/2), 58 (Hg 5p and Hg 5p3/2), 7 (Hg 5p, Hg 5d5/2, and Hg 5d3/2) [34] . It means that the possibility of mercury attached on the surface is not something of a concern.

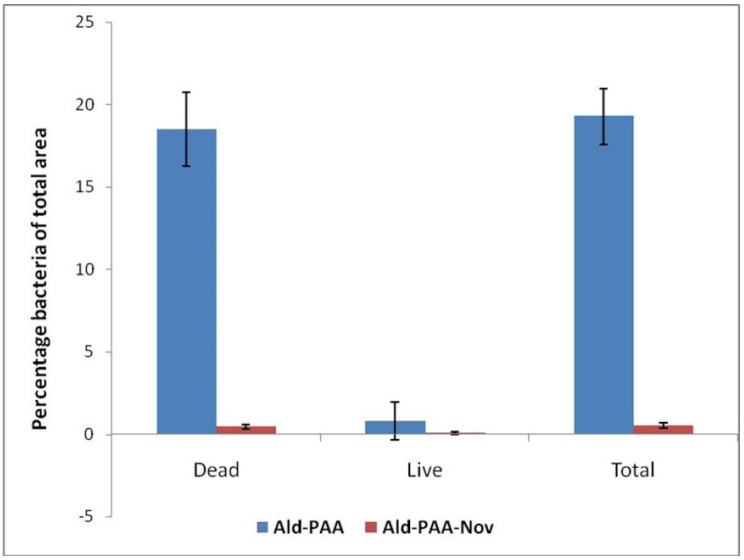

Fig. 5. Antibacterial activity of both layers before (Ald-PAA) and after (Ald-PAA-Nov) novobiocin immobilised.

The activity of novobiocin after being immobilised was still able to inhibit bacteria attachment. For the first hour incubation of both samples (before and after novobiocin immobilisation), bacteria attached on the surface. However, consistency of spots of negative control (before novobiocin immobilisation) spread much more than after novobiocin immobilisation. It is becoming significant after forth hour incubation, the spots spread bulky to negative control but consistency spot of after immobilisation were stagnant, even relatively the same as the similar sample of the first hour incubation.

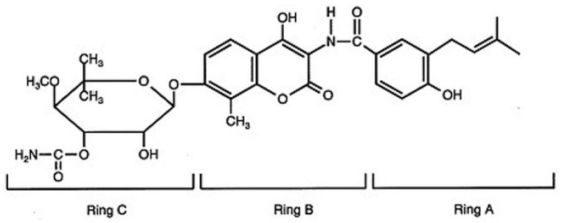

Fig. 6. Structure and region of novobiocin [30]

The small amount of bacteria attached on the surface after novobiocin immobilisation, presumably, settled on the delaminating surface antibacterial activity that F 1s was detected at the Ald-PAA-Nov layer as shown in Fig. 2. The F 1s was from the FEP as the base material –XPS data of FEP is not showed.

The effect of novobiocin activity, particularly, toward live or dead bacteria could not be explained yet. However, generally, the percentage reduction of total bacteria after novobiocin immobilised shown that the functional group of novobiocin as the active side to inhibit bacteria is effective and work as antibacterial coating on the surface.

5. Conclusion

The antibacterial of novobiocin immobilised covalently onto material surface. *In vitro* study shows that immobilised novobiocin has reduced bacterial activity significantly. Further treatment and assessment to avoid delaminating of interfacial layer become urgent to get the better surface antibacterial immobilised so that the surface activity maximise hindering bacterial attachment.

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